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(54) Title: METAL CHELATORS

(57) Abstract

Radionuclide chelating compounds are provided for coupling to targetting molecules such as proteins, peptides or antibodies. The resulting labelled targeting molecules may be used in diagnosis and therapy.

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METAL CHELATORS

Field of the Invention

This invention is in the field of diagnostic imaging, and relates to chemical chelators useful in the radiolabelling of agents that target tissues of diagnostic and therapeutic interest.

Background to the Invention

The art of diagnostic imaging exploits contrasting agents that in binding or localizing site selectively within the body, help to resolve the image of diagnostic interest. ^{67}Gallium-citrate, for example, has an affinity for tumours and inflamed tissue and, with the aid of scanning tomography, can reveal afflicted body regions to the physician. Other contrasting agents include the metal radionuclides such as ^{99m}technetium and $^{186/188}$rhenium, and these have been used to label targetting molecules, such as proteins, peptides and antibodies that localize at desired regions of the human body.

As targetting agents, proteins and other macromolecules can offer the tissue specificity required for diagnostic accuracy; yet labelling of these agents with metal radionuclides is made difficult by their physical structure. Particularly, protein and peptide targetting agents present numerous sites at which radionuclide binding can occur, resulting in a product that is labelled heterogeneously. Also, despite their large size, proteins rarely present the structural configuration most appropriate for high affinity radionuclide binding, i.e. a region incorporating four or more donor atoms that form five-membered rings. As a result, radionuclides are bound typically at the more abundant low-affinity sites, forming unstable complexes.

To deal with the problem of background binding, Paik et al (Nucl Med Biol 1985, 12:3) proposed a method whereby labelling of antibody is performed in the presence of excess DPTA (diaminetrimethylenepentaacetic acid), to mask the low affinity binding sites. While the problem of low affinity binding is alleviated by this method, actual binding of the radionuclide, in this case technetium, was consequently also very low. The direct labelling of proteins having a high proportion of cysteine residues also has been demonstrated (Dean et al; WO 92/13,572). This approach exploits thiol groups of cysteine residues as high-affinity sites for radionuclide binding, and is necessarily limited in application to those targetting agents having the required thiol structure.

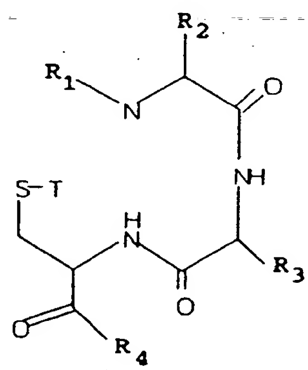
A promising alternative to the direct labelling of targetting agents is an indirect approach, in which targetting agent and radionuclide are coupled using a chelating agent. Candidates for use as chelators are those compounds that bind tightly to the chosen metal radionuclide and also have a reactive functional group for conjugation with the targetting molecule. For use in labelling peptide and protein-based targetting agents, the chelator is ideally itself peptide-based, to allow the chelator/targetting agent to be synthesized in any desired structural combination using peptide synthesis techniques. For utility in diagnostic imaging, the chelator desirably has characteristics appropriate for its *in vivo* use, such as blood and renal clearance and extravascular diffusibility.

Summary of the Invention

The present invention provides chelators that bind diagnostically useful metals, and can be coupled to targetting agents capable of localizing at body sites of

diagnostic and therapeutic interest. The chelators of the present invention are peptide analogues designed structurally to present an N_3S configuration capable of binding oxo, dioxo and nitrido ions of radionuclides such as ^{99m}technetium and $^{186/188}$rhenium.

More particularly, and according to one aspect of the invention, there are provided metal chelators of the formula:



wherein

R_1 and R_2 together form a 5- or 6-membered heterocyclic ring which is optionally fused to a 5- or 6-membered ring, wherein either ring is optionally substituted with a conjugating group or with a conjugating group having a targetting molecule coupled thereto;

R_3 is selected from H; alkyl; and alkyl substituted by a group selected from amino, aminoacyl, carboxyl, guanidinyl, hydroxyl, thiol, phenyl, phenolyl, indolyl and imidazolyl;

R_4 is selected from hydroxyl; alkoxy; an amino acid residue; and a targetting molecule; and

T is H or a sulfur protecting group;

In an aspect of the invention, chelators of the above formula are provided in a form having a diagnostically or therapeutically useful metal complexed therewith.

According to another aspect of the invention, the chelator is provided in a form coupled to a diagnostically or therapeutically useful targetting molecule. An additional aspect of the invention provides the chelators coupled to a targetting molecule and in a form having a metal complexed therewith.

In another aspect of the invention, targetting molecules are provided having the general sequence: formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH wherein X is a bond or an amino acid residue; the targetting molecule which may be coupled to chelators of the present invention.

Brief Description of the Figures

Figure 1 is a graph representing binding affinity of targetting molecules in accordance with an embodiment of the invention.

Figure 2 is a graph representing neutropenic effect of targetting molecules in accordance with an embodiment of the invention.

Detailed Description of the Invention

The invention provides chelators of diagnostically useful metals that when complexed with the metal and in a form coupled to a targetting molecule are useful for delivering the detectable metal to a body site of diagnostic interest. As illustrated in the above formula, the chelators are peptidic derivatives that

present an N_3S configuration in which the metal is complexed.

Terms defining the variables R_1 - R_4 and T as used hereinabove have the following meanings:

"alkyl" refers to a straight or branched C_1-C_8 chain and includes lower C_1-C_4 alkyl;
"alkoxy" refers to straight or branched C_1-C_8 alkoxy and includes lower C_1-C_4 alkoxy;
"thiol" refers to a sulfhydryl group that may be substituted with an alkyl group to form a thioether;
"sulfur protecting group" refers to a chemical group that inhibits oxidation of sulfur and includes groups that are cleaved upon chelation of the metal. Suitable sulfur protecting groups include known alkyl, aryl, acyl, alkanoyl, aryloyl, mercaptoacyl and organothio groups.

In preferred embodiments of the invention, the chelators conform to the above formula in which: R_1 and R_2 together form a five or six membered heterocyclic ring such as pyrrole and pyridine, or a five or six membered ring fused to a six membered ring such as indole, quinoline and isoquinoline; R_3 is selected from H and a hydroxy substituted alkyl group selected from methyl and ethyl and most preferably hydroxymethyl; R_4 is selected from hydroxyl; alkoxy; an amino acid residue; and a targetting molecule; and T is a hydrogen atom or the sulfur protecting group acetamidomethyl (Acm);

In specific embodiments of the invention, the chelators conform to the above general formula wherein T is the sulfur protecting group acetamidomethyl (Acm); R_3 is H or hydroxymethyl; R_1 and R_2 together form a ring selected from pyridine, pyrrole, indole, quinoline and

isoquinoline; and R_4 is a glycine amino acid residue or a glycine residue attached to a targetting peptide.

The substituents represented by R_1 and R_2 together with the adjacent nitrogen atom form a 5- or 6-membered heterocyclic ring which may be fused to another five or six membered ring. Five and six membered heterocyclic rings include but are not limited to pyrrole, pyrazole, imidazole, pyridine, pyrazine, pyridazine, pyrimidine and triazine. Fused rings include but are not limited to N-containing bicyclics such as quinoline, isoquinoline, indole and purine. Rings containing sulfur atoms e.g. thiazole and oxygen atoms e.g. oxazole are also encompassed by the present invention.

The heterocyclic ring formed by R_1 and R_2 may be substituted with a conjugating group that is chemically reactive allowing for coupling a targetting molecule to the chelator. In the preferred case where the targetting molecule is peptidic, the conjugating group is reactive under conditions that do not denature or otherwise adversely affect the peptide. In one embodiment of the invention, the conjugating group is reactive with a functional group of the peptidic targetting molecule such as the carboxy terminus or amino terminus. Alternatively, the conjugating group can be reactive with an ϵ-amino group of a lysine residue. Conjugating groups reactive with amino groups of targetting molecules include carboxyl and activated esters. Conjugating groups reactive with carboxyl groups of targetting molecules include amines and hydrazines.

For diagnostic and therapeutic purposes, the chelator per se may be used in combination with a detectable metal capable of forming a complex. Suitable metals include radionuclides such as technetium and rhenium in their various forms such as $^{99m}TcO^{3+}$, $^{99m}TcO_2^+$, ReO^{3+} and ReO_2^+.

More desirably, the chelator is coupled to a targetting molecule that serves to localize the chelated metal to a desired location for diagnostic imaging or for therapy ie. radiation therapy of tumours. Examples of targetting molecules include, but are not limited to, steroids, proteins, peptides, antibodies, nucleotides and saccharides. Preferred targetting molecules include proteins and peptides, particularly those capable of binding with specificity to cell surface receptors characteristic of a particular pathology. For instance, disease states associated with over-expression of particular protein receptors can be imaged by labelling that protein or a receptor binding fragment thereof in accordance with the present invention. Peptide-based targetting molecules can be made by various known methods or in some instances can be commercially obtained. Solid phase synthesis employing alternating t-Boc protection and deprotection is the preferred method of making short peptides which can be an automated process. Recombinant DNA technology is preferred for producing proteins and long fragments thereof.

Chelators of the present invention are peptide derivatives and are most efficiently prepared by solid-phase peptide synthesis. In general solid-phase synthesis involves the stepwise addition of amino acid residues to a growing peptide chain that is linked to an insoluble support or matrix, such as polystyrene. The C-terminus residue of the chelator is first anchored to a commercially available support with its amino group protected with an N-protecting agent such as a t-butyloxycarbonyl group (tBoc) or a fluorenylmethoxycarbonyl (FMOC) group. The amino protecting group is removed with suitable deprotecting agents such as TFA in the case of tBOC or piperadine for FMOC and the next amino acid residue (in N-protected form) is added with a coupling agent such as

dicyclocarbodiimide (DCC). Upon formation of a peptide bond the reagents are washed from the support. After addition of the final residue, the chelator is cleaved from the support with a suitable reagent such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF) and isolated.

The present invention encompasses chelators incorporating various heterocyclic groups containing a nitrogen atom provided that it is analogous in structure to an amino acid in that there is a carboxyl carbon, alpha carbon and an alpha nitrogen wherein the alpha carbon and alpha nitrogen are incorporated in a common ring. For example, picolinic acid (pic), dipicolinic acid (dipic), chelidamic acid (chel), 2-carboxypyrazine, 2-carboxypyrimidine, 2-carboxypyrrole, 2-quinolinic acid, 1-isoquinolinic acid, 3-isoquinolinic acid and the like will behave as a natural amino acid residue in solid phase synthesis by forming a peptidic bond upon reaction of the carboxyl group and a deprotected amino group of a previously added residue. Variation at R_3 may be introduced to chelators of the invention simply by incorporation of a desired amino acid residue at the appropriate stage of chain elongation. For example, R_3 may be a hydroxymethyl group by using a serine residue or may be a hydrogen atom by using glycine. Any D or L, naturally occurring or derivatized amino acid may be used.

In accordance with an embodiment of the present invention, R_4 is a targetting molecule that is proteinaceous. Human Immunoglobulin G (HIG), a multisubunit protein, has been directly labelled with technetium-99m and used extensively for imaging sites of inflammation, however smaller peptides are becoming the targetting molecules of choice for their site specificity as a result of receptor binding properties and for their

ease of preparation. An example of peptidic targetting molecules are Tuftsin antagonists such as Thr-Lys-Pro-Pro-Arg and Lys-Pro-Pro-Arg. Another peptidic targetting molecule useful for imaging inflammation is fMLP (formyl-Met-Lys-Phe) and derivatives thereof such as formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys described by Fischman et al in pending Canadian application CA 2,016,235. It is believed that fMLP and various derivatives thereof bind to neutrophils and are therefore useful in imaging sites of inflammation.

In accordance with another aspect of the invention, the present invention provides a peptide useful as a targetting molecule which has the sequence formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH wherein X is a bond or an aminoacid residue. For convenient synthesis of this peptide, X is preferrably a glycine (Gly) residue. In vivo studies have shown this peptide coupled to a chelator of the present invention strongly binds to neutrophils while having a more favourable neutropenic profile than native fMLP or the derivative formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys.

Synthesis of a chelator/targetting molecule conjugate hereinafter referred to as a "conjugate" can be achieved in various ways. When R_4 is a peptidic targetting molecule, it is convenient to synthesize the conjugate *in toto* by starting solid-phase synthesis from the C-terminus residue of the targetting molecule and ending with the heterocyclic residue (R_1, R_2) of the chelator. Alternatively, a targetting molecule which incorporates a lysine residue may be coupled to the chelator at R_4 by way the ε-amino group of that lysine residue. In this case, the targetting peptide is synthesized as a separate chain from the chelator and is differentially protected at the ε-amino group and N-terminus amino group. For example the ε-amino group may be protected with 1-(4,4-dimethyl-

2,6-dioxocyclohexylidine)-ethyl (Dde) while the N-terminus amino is FMOC protected. When the targetting molecule synthesis is complete the ε-amino group is deprotected with hydrazine and is available for reaction with a C-terminus carboxyl group of a chelator while the N-terminus amino group is protected.

Targetting molecules may also be coupled to chelators of the invention by way of a conjugating group substituent on the heterocyclic ring of the chelator. For example, a chelator with an amino substituent on the heterocyclic ring upon deprotection will be reactive with the C-terminus carboxyl group of a peptide targetting molecule. Such a conjugate may be synthesized as a single chain starting at the C-terminus residue of the chelator and ending with the N-terminus of the targetting molecule. Alternatively, a peptide targetting molecule may be coupled to the heterocyclic residue by way of its N-terminus when the heterocyclic group has a suitable conjugating group substituent such as a carboxyl group or an activated ester. In this case, the chelator and targetting molecule are synthesized as separate chains and then coupled to form the desired conjugate.

In accordance with one aspect of the invention, chelators incorporate a diagnostically or therapeutically useful metal. Incorporation of the metal within the chelator can be achieved by various methods common to the art of coordination chemistry. When the metal is the radionuclide technetium-99m, the following general procedure may be used to form a technetium complex. A chelator solution is formed initially by dissolving the chelator in aqueous alcohol eg. ethanol-water 1:1. The solution is degassed with nitrogen to remove oxygen then the thiol protecting group is removed, for example with sodium hydroxide and heat. The solution is then neutralized with an organic acid such as acetic acid (pH

6.0-6.5). In the labelling step, sodium pertechnetate, obtained from a Molybdemun generator, is added to the chelator solution with an amount of stannous chloride sufficient to reduce the technetium. The solution is mixed and left to react at room temperature and then heated on a water bath. In an alternative method, labelling can be accomplished with the chelator solution adjusted to pH 8. Pertechnetate may be replaced with a solution of technetium complexed with labile ligands suitable for ligand exchange reactions with the desired chelator. Suitable ligands include tartarate, citrate, gluconate and glucoheptonate. Stannous chloride may be replaced with sodium dithionite as the reducing agent if the chelating solution is alternatively adjusted to pH 12-13 for the labelling step. The labelled chelator may be separated from contaminants $^{99m}TcO_4^-$ and colloidal $^{99m}TcO_2$ chromatographically, e.g. with a C-18 Sep Pak cartridge activated with ethanol followed by dilute HCl. Eluting with dilute HCl separates the $^{99m}TcO_4^-$, and eluting with EtOH-saline 1:1 brings off the chelator while colloidal $^{99m}TcO_2$ remains on the column. The chelators of the invention can be coupled to a targetting molecule prior to labelling with the radionuclide, a process referred to as the "bifunctional chelate" method. An alternative approach known as the "prelabelled ligand" method, the chelator is first labelled with the desired metal and is subsequently coupled to the targetting molecule. This method is advantageous in that the targetting molecule itself is not inadvertently labelled at low affinity binding sites which may render the targetting molecule inactive or may release the metal in vivo.

An alternative approach for labelling chelators of the present invention involves techniques described in a co-pending U.S. application 08/152,680 by Pollak et al, filed on 16 November 1993 incorporated herein by reference. Briefly, chelators are immobilized on a solid

phase support in such a manner that they are released from the support only upon formation of a complex with the labelling metal atom. This is achieved when the chelator is coupled to a functional group of the support by one of the complexing atoms. Preferably, a complexing sulfur atom is coupled to the support which is functionalized with sulfur a protecting group such as maleimide.

When coupled to a targetting molecule and labelled with a diagnostically useful metal, chelators of the present invention can be used to detect pathological conditions by techniques common in the art. A conjugate labelled with a radionuclide metal such as technetium may be administered to a mammal by intravenous injection in a pharmaceutically acceptable solution such as saline or DMSO. The amount of labelled conjugate administered is dependent upon the toxicity profile of the chosen targetting molecule as well as the metal. Localization of the metal in vivo is tracked by standard scintigraphic techniques at appropriate time intervals subsequent to administration.

The following examples are presented to illustrate certain embodiments of the present invention.

<u>Example 1</u> **Preparation of Chelators and Conjugates**

Chelators were synthesized using 9-fluorenylmethyloxycarbonyl (FMOC) chemistry on an 2-methoxy-4-alkoxybenzyl alcohol resin preloaded with the protected C-terminus residue (Sasrin resin, Bachem Biosciences Inc., Philadelphia PA) using an Applied Biosystems 433A peptide synthesizer (Foster City, CA).

Preparation of Chelators

a. Chel-Gly-Cys(Acm)-Gly-OH

b. DiPic-Gly-Cys(Acm)-Gly-OH

c. Pic-Gly-Cys(Acm)-Gly-OH

Synthesis began from the Gly residue preloaded on the resin and continued to the final Pic, DiPic or Pic residue by addition of one of picolinic, dipicolinic or cheladamic acid. The chelator-resin was dried in vacuo for 12 hours. Cleavage of the chelator from the resin involved mixing a cooled solution of 95% trifluoroacetic acid (TFA) and 5% water (1ml per 100 mg of peptide-resin) with the peptide-resin for 1.5 to 2 hours at room temperature. The resin was removed by filtration and washed 3 times with 30 ml t-butyl methyl ether in a 50 ml conical polypropylene centrifuge tube forming a white precipitate. The precipitate was dissolved in water with added acetonitrile. The precipitate was frozen in acetone-dry ice and lyophilized over 12 hours. The resulting white powder was dissolved in water, filtered through a 0.45 μm syringe filter (Gelman Acrodisc LC PVDF) and purified by reversed-phase HPLC (Beckman System Gold) with a C18 column (Waters RCM 25 x 10) using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The column was equilibrated with 100:0 buffer A:buffer B and eluted with a linear gradient in 25 min at 1 ml/min to 50% buffer B. Fractions were reanalysed on the HPLC and pooled according to matching profiles. If necessary the pooled fractions were repurified using the same conditions. The pure fractions were frozen in acetone-dry ice and lyophilized over 10 hours to give a white powder.

Preparation of Conjugates

d. (Pic-Ser-Cys(Acm)-Gly)-Thr-Lys-Pro-Pro-Arg-OH;

e. (Pic-Ser-Cys(Acm)-Gly)-Lys-Pro-Pro-Arg-OH;

f. 2-Quinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH;

g. 1-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH;

h. 3-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH;

i. Indole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH; and

j. Pyrrole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH.

Synthesis began from the Arg residue preloaded on the resin and continued to the Ser residue of the chelator ending with the addition of one of picolinic, 2-quinolinic, 1-isoquinolinic, 3-isoquinolinic, pyrrole-2-carboxylic and indole-2-carboxylic acid. The chelator-peptide-resin was dried in vacuo 12 hours. Cleavage from the resin involved mixing with a solution of 10 ml trifluoroacetic acid (TFA), 0.5ml water, 0.5ml thioanisole, 0.25ml 1,2-ethanedithiol (EDT) and 0.75g phenol for 1.5 to 2 hours at room temperature. The resin was removed by filtration and the peptide washed 3 times with 30 ml t-butyl methyl ether in a 50 ml conical polypropylene centrifuge tube forming a white precipitate. The precipitate was dissolved in water with added acetonitrile when solubility problems arose. The precipitate was frozen in acetone-dry ice and lyophilized over 12 hours. The resulting white powder was dissolved in water, filtered through a 0.45 μm syringe filter (Gelman Acrodisc LC PVDF) and purified by reversed-phase HPLC (Beckman System Gold) with a C18 column (Waters RCM 25 x 10) using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The column was equilibrated with 100:0 buffer A:buffer B and eluted with a linear gradient in 25 min at 1 ml/min to 50% buffer B. Fractions were reanalysed on the HPLC and pooled according to matching profiles. If necessary the pooled fractions were repurified using the same conditions. The

pure fractions were frozen in acetone-dry ice and lyophilized over 10 hours to give a white powder.

Preparation of fMLP Conjugates

k. (Pic-Gly-Cys-Gly)-εNH-Lys(-Gly-OH) -Tyr-Nleu-Phe-Leu-Nleu-for

l. (Pic-Gly-Cys(Acm)-Gly)-εNH-Lys(-Gly-OH) -Tyr-Nleu-Phe-Leu-Nleu-for

m. (Pic-Gly-Cys(Acm)-Gly)-ε-NH-Lys(-Asp-Gly-OH)-Lys-Tyr-Nleu-Phe-Leu-Nleu-for

Targetting peptides that comprise lysine residues can be coupled to the chelator via the Lys ε-amino group by the following procedure. For compounds example 1(k), 1(l), and 1(m). the targetting peptide was initially synthesized from glycine to norleucine by 9-fluorenylmethyloxycarbonyl (FMOC) chemistry using an FMOC-glycine preloaded 2-methoxyl-4-alkoxyl-benzyl alcohol resin and a 1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-ethyl (Dde) orthogonal protected lysine with an Applied Biosystems 433A peptide synthesizer. The fMLP peptide-resin was removed from the synthesizer and dried 12 hours in vacuo to prepare for formylation.

Formic anhydride was prepared by heating acetic anhydride (2 equivalents) with formic acid (1 equivalent) to 50°C for 15 minutes followed by cooling to 0°C. Formylation of the fMLP peptide involved swelling the peptide-resin in dichloromethane (DCM) (5 ml) followed by swirling with formic anhydride (5ml) for 15 minutes. The formylated fMLP peptide-resin was filtered, washed with DCM and dried in vacuo 12 hours.

Formylated peptide-resin (50 mg/2ml) was swirled with a 2% hydrazine hydrate in N-methylpyrrolidone (NMP)

solution for 3 minutes two times then filtered and washed with DCM and dried in vacuo 12 hours to remove the ε-amino lysine protecting group (Dde) while leaving the N-terminus amino group formylated.

The chelator was added to the ε-amino lysine of the fMLP peptide on the 433A peptide synthesizer. The chelator-peptide-resin was dried in vacuo 12 hours. Cleavage from the resin involved mixing a cooled solution of 95% trifluoroacetic acid (TFA) and 5% water (1 ml per 100 mg of chelator-peptide-resin) with the chelator-peptide-resin for 1.5 to 2 hours at room temperature. The resin was removed by filtration and washed with 1-3 ml of TFA to obtain 6-8 ml of a clear yellow liquid. This liquid was slowly dropped into 30-35 ml of tert-butyl methyl ether in a 50 ml conical polypropylene centrifuge tube forming a white precipitate. The precipitate was centrifuged at 7000 rpm, 0°C for 5 minutes (Sorvall RT 6000, Dupont), decanted and washed two more times with t-butyl methyl ether. Following drying under vacuum the precipitate was dissolved in water with added acetonitrile when solubility problems arose. The precipitate was frozen in acetone-dry ice and lyophilized over 10 hours. The resulting white powder was dissoved in dimethylsulfoxide (20 μL) and 50:50 acetonitrile:water solution (980 μL), filtered through a 0.45 μm syringe filter (Gelman Acrodisc LC PVDF), and purified by reversed-phase HPLC (Beckman System Gold) with a C18 column (Waters RCM 25 X 10) using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The column was equilibrated with 50:50 buffer A:buffer B and eluted with a linear gradient in 25 min at 1 ml/min to 100% buffer B. Fractions were reanalysed on the HPLC and pooled according to matching profiles. If necessary, the pooled fractions were repurified using the same conditions. The pure fractions were frozen in acetone-

dry ice and lyophilized over 12 hours to give a white powder.

Example 2 Labelling Chelators with ^{99m}Tc

The chelators and conjugates of example 1 (1mg) were dissolved in 200μL EtOH-water (1:1) in a tube. 100-200μL sodium pertechnetate (200-600 MBa, 5-15 mCi), 100 μL phosphate buffer (0.25 M, pH 7.4), and 200 μL of a solution containing 50μg stannous chloride dihydrate and 40mg sodium tartrate were added to the tube and capped tightly and placed in a boiling water bath for 10 minutes. In order to achieve adequate separation of the chelators, the solution was then loaded on a C-18 Sep-Pak column activated by washing sequentially with 5ml methanol, 10ml water and 5ml dilute (1mM) HCl to remove TcO_4^-. Subsequent elution with 2ml EtOH-saline (1:1) removed the chelator while TcO_2 remained on the column. The extent of complexation of ^{99m}Tc with chelators was measured by radioactivity of the eluted fractions.

Example	Labelling Yield (% total radioactivity)
1(a)	7
1(c)	92
1(d)	93
1(e)	92
1(k)	29
1(l)	63
1(m)	78

Conjugates 1(f) - 1(j), were reconstituted (200μL, 1mg/mL saline) and then injected into 3mL vacutainers with 100μL pertechnetate (10mCi) and 100μL stannous gluconate (50 μg stannous chloride and 1 mg sodium gluconate). The tubes were placed in boiling water bath for 12 minutes and then filtered through a Whatman PVDF syringe filter to collect the labelled conjugate solutions which were further diluted with saline to prepare injectable solutions (2Mbq/mL). The conjugates were isolated by HPLC (Beckman) from a (20μL) sample (before dilution) to determine the labelling yield by measuring radioactivity.

example	Labelling Yield (%)
1(f)	94.4
1(g)	96.8
1(h)	94
1(i)	96.3
1(j)	98.4

Example 3 In vivo imaging and biodistribution of chelators and conjugates

For chelators and conjugates of example 1, rat inflammation studies were performed as follows. 2 male Sprague-Dawley rats (Charles River, 250-300g) were injected intramuscularly with 5mg zymosan, a yeast cell wall preparation (20mg for conjugates 1(f) - 1(i)) or a virulent E.coli (ATCC 25922, 0.1ml of $1.0X10^9$ organisms/ml) suspension into their right hindlegs 24 hours before imaging. Focal inflammation in the leg was visually detectable after 1 day. 1mg (ca. 0.7 μMol) of the chelator was dissolved in 50 μL of dimethylsulfoxide and added to an ethanol-water mixture (1:1, 200 μL). An aliquot of Tc-99m tartarate (ca. 400 MBq) was added and

transchelation allowed to proceed for 20 min. at 100°C. The Tc-99m chelate was purified by elution through a Sep Pak cartridge. The purified tracer solution was further diluted with saline to prepare an injectable (200 μL) containing about 100 μCi (3.7 MBq) of activity.

The rats were anaesthetized with sodium pentobarbital (40 to 50 mg/kg), and the labelled chelator/conjugate solution (200μL) was injected intravenously via the tail vein. Serial whole-body scintigrams were acquired for the first 5 minutes. Subsequently, further images were obtained at 30, 60, and 120 minutes. The rats were then killed with anaesthesia and samples of organs, urine, blood, inflamed muscle (right leg) and non-inflamed muscle (left leg) were weighed and counted in either a well-type gamma counter or in a gamma dose calibrator. The dose calculations were made based on assumption that the blood volume constituted 6.5% of body weight. Results are averages for two rats and are corrected for the residual dose in the tail.

In vivo Distribution

example	blood	liver	kidney	urine	GI tract	time (min)	Inflam muscle	Uninfl muscle	Inflam agent	Infl : Uninfl	time (min)
	(% per organ)						(% per g)				
1(a)	1.016	0.953	4.359	33.950	52.619	88			none		
1(c)	0.187		21.700				0.077	0.019	E.coli	4.24	
1(d)	6.370	3.050	4.170	44.710	5.080	30	0.180	0.050	E.coli	3.62	30
1(e)	3.520			53.000		30	0.125	0.059	E.coli	2.1	
1(f)	5.360	4.731	4.037	51.372	4.416	35	0.095	0.025	zymosan	4.8	35
1(g)	5.154	3.219	6.656	43.783	7.164	35	0.132	0.030	zymosan	4.4	35
1(h)	5.187	3.155	3.077	58.460	4.117	35	0.101	0.030	zymosan	3.7	35
1(i)	8.674	4.989	2.330	40.274	39.179	35	0.177	0.035	zymosan	5.1	35
1(k)	3.036	35.26	18.82	8.400	41.285	90			zymosan		
1(l)	3.436	20.866	11.470	14.050	25.777	109			E.coli	3.17	
1(m)							0.143	0.041	E.coli	3.52	45
IgG	48					120	0.104	0.052	E.coli	2.07	120

Example 4 Neutrophil Binding Assay of fMLP and fMLP Derivative Conjugates of Example 1(l) and 1(m)

Rat peripheral neutrophils were prepared for binding assay as follows: blood was obtained by cardiac puncture and anticoagulated with acid- citrate dextrose (ACD) (10%). Red blood cells were removed by sedimentation on hydroxyethyl cellulose (1.1%) for 30 min at room temperature and leukocyte-rich supernatant layered onto 65% percol. Centrifugation at 400g for 30 min resulted in a distinct band of mononuclear cells (lymphocytes and monocytes) which was discarded, the neutrophil rich pellet was resuspended and remaining red blood cells lysed by hypotonic shock using cold water. The remaining neutrophils were resuspended in Hanks Buffered Salt Solution (HBSS) to the desired concentration. Final neutrophil preparation consisted of cells pooled from up to 10 animals, >90% neutrophils and >95% viable by Trypan Blue exclusion.

Binding affinity of the fMLP peptide and conjugates 1(l) and 1(m) was assessed by competing off a constant concentration of tritiated fMLP of known affinity for neutrophil receptors. 10^6 neutrophils were added to polypropylene plates containing 15nM tritiated fMLP and varying concentrations of unlabelled test peptide and conjugates in a final volume of 150μL HBSS. The plate was incubated for 1 hour at room temperature after which cells were harvested by filtration onto glass fibre filter mats (Skatron receptor binding filtermat) using a Skatron cell harvester with 12 well head. Harvested cells were washed with ice-cold saline and air dried. Filters were then placed in 6ml scintillation vials, 5ml of scintillation fluid added (Ecolume) and vials counted using a liquid scintillation counter. Binding affinity of the fMLP peptide and conjugates 1(l) and 1(m) is illustrated in figure 1 and expressed as % maximal

tritiated fMLP binding vs. peptide/conjugate concentration. % maximal tritiated fMLP binding=(specific binding ÷ maximum binding) X 100%. Specific binding was the total binding less non-specific binding which was the amount of residual radioactivity bound in the presence of 10μM unlabelled fMLP. Both 1(l) and 1(m) had greater binding affinity for neutrophil receptors than native fMLP.

Example 5 Neutropenia Assay of fMLP, fMLP Derivative formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys and Conjugate 1(m)

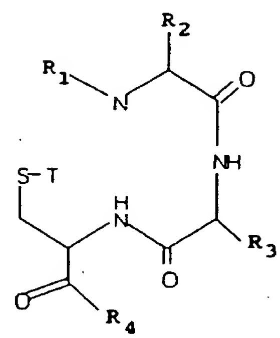
The effect of fMLP, fMLP derivative formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys and conjugate of example 1(m) on circulating neutrophil number was assessed using the rat transient neutropenia model. Rats were anaesthetized with 250μL somnitol (16mg/rat) and injected via the tail vein at T=0 with the test peptides. At a range of time points after injection (0, 2, 5, 10, 30 min) a 2ml blood sample was taken by cardiac puncture (anticoagulated with 10% ACD). 3 animal were used per time point. For each sample the total white blood cells/ml and % neutrophils was determined, the number of neutrophils/ml in each sample being calculated. Within each experiment the number neutrophils/ml after saline injection at all time points was meaned to give a saline control against which the peptides could be compared. The number neutrophils/ml after peptide injection was expressed as a % of the saline control within each experiment.

Referring to figure 2, injections of 5 and 10 nmoles of fMLP produced a dose-dependent transient neutropenia, with a maximal effect occurring 2 min after peptide injection (15 and 9% of control respectively) returning to 93 and 75% of control values by 30 min after injection. 5nmoles of formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys produced a smaller maximal reduction in circulating

neutrophils (45% of control) while 1(m) produced only a small transient drop in circulating neutrophils (80% of control) at 5nmoles.

WE CLAIM:

1. A compound of the general formula:



wherein

R_1 and R_2 together form a 5- or 6-membered heterocyclic ring which is optionally fused to a 5- or 6-membered ring, wherein either ring is optionally substituted with a conjugating group or with a conjugating group having a targetting molecule coupled thereto;

R_3 is selected from H; alkyl; and alkyl substituted by a group selected from amino, aminoacyl, carboxyl, guanidinyl, hydroxyl, thiol, phenyl, phenolyl, indolyl and imidazolyl;

R_4 is selected from hydroxyl; alkoxy; an amino acid residue; and a targetting molecule; and

T is H or a sulfur protecting group;

2. A compound according to claim 1, wherein the ring formed by R_1 and R_2 is a five or six membered heterocyclic ring optionally fused to a benzene ring.

3. A compound according to claim 1, wherein R_1 and R_2 together form a ring selected from pyridine, quinoline, isoquinoline, pyrrole and indole.

4. A compound according to claim 1, wherein R_1 and R_2 together form a ring selected from 6-carboxypyridine and 4-hydroxy-6-carboxypyridine.

5. A compound according to claim 1 wherein R_3 is selected from H and hydroxymethyl.

6. A compound according to claim 1, wherein R_4 is selected from -Gly-OH and -Gly-targetting molecule.

7. A compound according to claim 6, wherein the targetting molecule is a peptide.

8. A compound according to claim 7, wherein the peptide has a sequence selected from -NH-Lys-Pro-Pro-Arg-OH; and -NH-Thr-Lys-Pro-Pro-Arg-OH.

9. A compound according to claim 7, wherein the Gly forms an amide linkage with an ε-amino Lys residue of the peptide selected from:

-ε-amino Lys(-Gly-OH)-Tyr-Nleu-Phe-Leu-Nleu-formyl; and

-ε-amino Lys(-Asp-Gly-OH)-Lys-Tyr-Nleu-Phe-Leu-Nleu-formyl.

10. A compound according to claim 1, wherein R_1 and R_2 together form a pyridine ring; R_3 is H; T is Acm; and R_4 is selected from:

-Gly-OH; and

-Gly-ε-amino Lys(-Gly-OH)-Tyr-Nleu-Phe-Leu-Nleu-formyl.

11. A compound according to claim 1, wherein R_1 and R_2 together form a pyridine ring; R_3 is hydroxymethyl; T is Acm; and R_4 is selected from:

-Gly-OH;

-Gly-Lys-Pro-Pro-Arg-OH;

-Gly-Thr-Lys-Pro-Pro-Arg-OH;

-Gly-ε-amino Lys(-Gly-OH)-Tyr-Nleu-Phe-Leu-Nleu-formyl; and

-Gly-ε-amino Lys(-Asp-Gly-OH)-Lys-Tyr-Nleu-Phe-Leu-Nleu-formyl.

12. A compound according to claim 1, selected from:

2-Quinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH;

1-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH;

3-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH;

Pyrrole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH; and

Indole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-Arg-OH.

13. A compound according to claim 1, wherein R_1 and R_2 together form a ring selected from a 6-carboxypyridine ring and a 6-carboxy-4-hydroxypyridine ring; R_3 is hydroxymethyl; T is Acm; and R_4 is -Gly-OH.

14. A compound according to claim 1, in a form complexed with a metal or an oxide or nitride thereof.

15. A compound according to claim 10, wherein the metal is ^{99m}Tc.

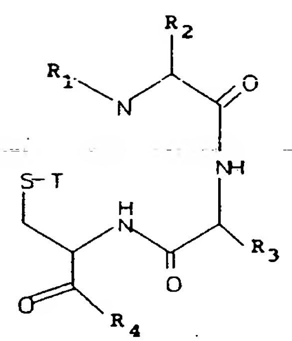
16. A compound according to claim 11, in a form complexed with ^{99m}Tc.

17. A compound according to claim 12, in a form complexed with ^{99m}Tc.

18. A method of imaging for sites of in vivo localization of a targetting molecule comprising the steps:

1) administering a diagnostically effective amount of a compound according to claim 15, comprising said targetting molecule; and
2) detecting localization of the compound.

19. A compound of the general formula:



wherein:

R_1 and R_2 together form a 5- or 6-membered heterocyclic ring which is optionally fused to a 5- or 6-membered ring, wherein either ring is optionally substituted with a conjugating group or with a conjugating group having a targetting molecule coupled thereto;

R_3 is selected from H; alkyl; and alkyl substituted by a group selected from amino, aminoacyl, carboxyl, guanidinyl, hydroxyl, thiol, phenyl, phenolyl, indolyl and imidazolyl;

R_4 is selected from hydroxyl; alkoxy; an amino acid residue; and a targetting molecule; and

T is H or a sulfur protecting group;

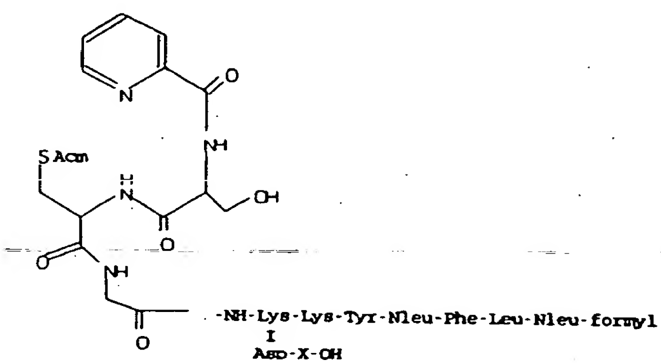
wherein the targetting molecule is a peptide having a sequence:

formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH

and X is a bond or an amino acid residue.

20. A compound according to claim 19, wherein X is -Gly-.

21. A compound according to claim 19, having the formula:



wherein X is a bond or an amino acid residue.

22. A compound according to claim 21, wherein X is -Gly-.

23. A peptide having the general sequence:

formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH

wherein X is a bond or an amino acid residue.

24. A peptide according to claim 23, wherein X is -Gly-.

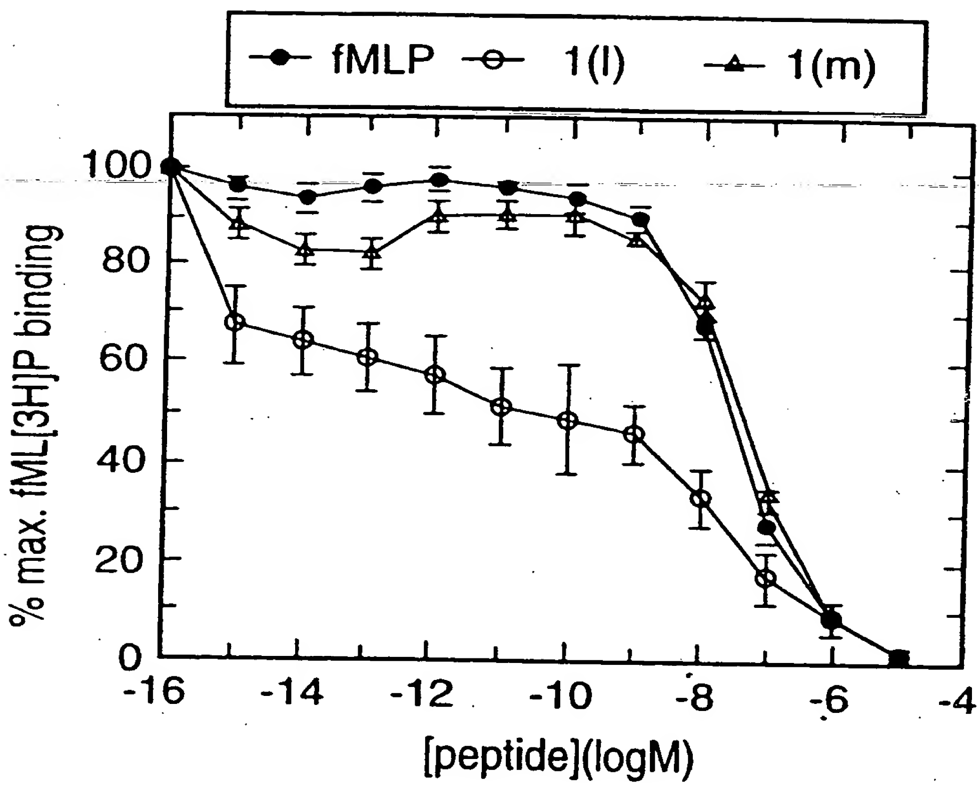

fMLP
1(l)
1(m)
100
80
60
40
20
0
% max. fML[3H]P binding
-16
-14
-12
-10
-8
-6
-4
[peptide](logM)

FIG.1

2/2

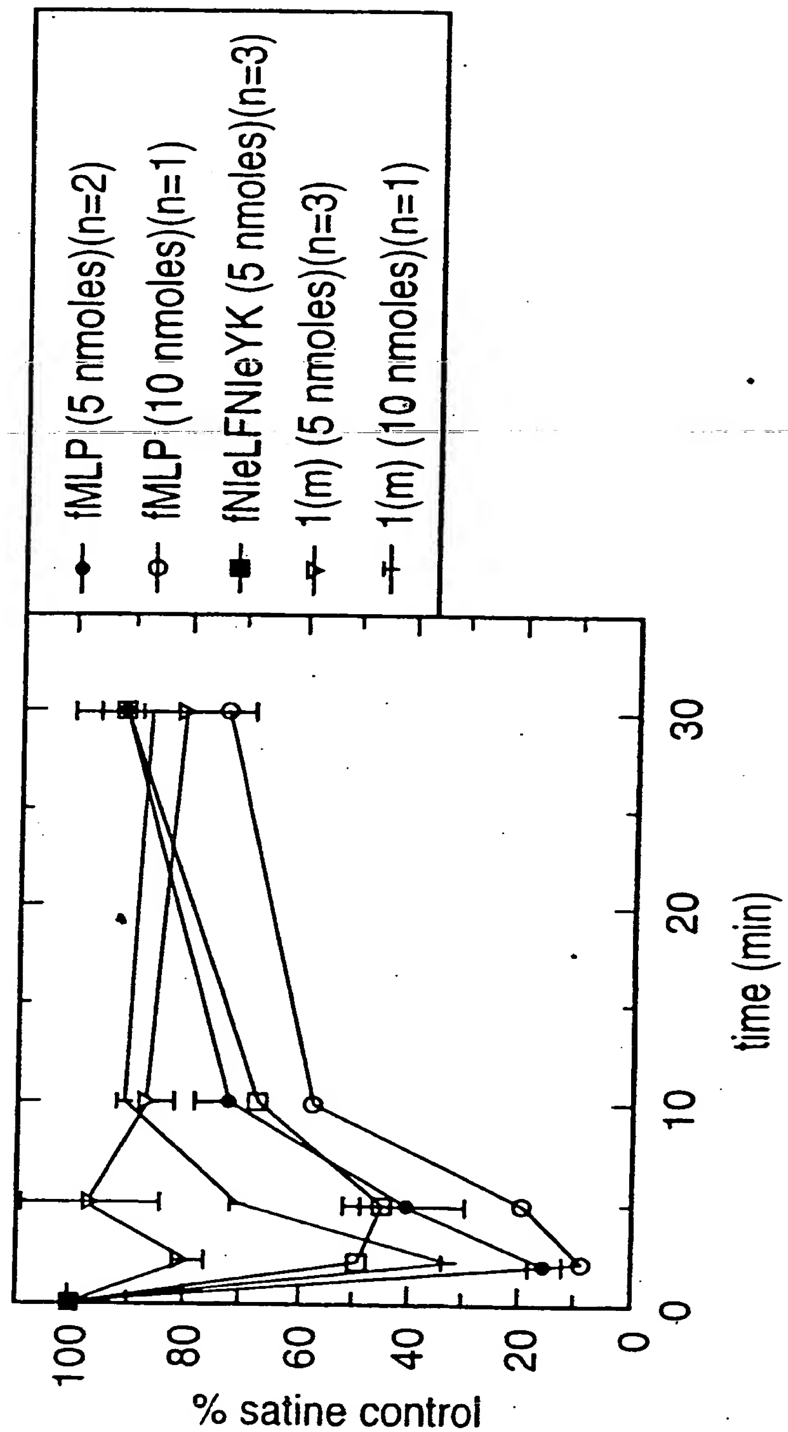


FIG.2.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No
PCT/CA 94/00718

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K5/097 C07K5/06 A61K51/08 A61K51/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 23085 (DIATECH,INC.) 25 November 1993	1-7,14
Y	see page 9, line 16 - page 10, line 6 see page 17, line 18 - line 22 see page 49; claim 59 ---	1-7,9-24
X	INT.J.PEPTIDE PROTEIN RES., vol. 5, 1973 pages 91-98, AKHTAR A. ET AL. 'SYNTHESIS OF A CHELATED CORE RELATED TO RUBREDOXIN' see example X ---	1-3
X	WO,A,93 22338 (RIJKSUNIVERSITEIT LEIDEN) 11 November 1993 *peptid PLCDLLIRC* see claim 5 ---	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1995	11-05-1995
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Deffner, C-A

International application No
PCT/CA 94/00718

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 398 143 (THE GENERAL HOSPITAL CORPORATION) 22 November 1990 see the whole document ---	1-7,9-24
A	US,A,4 687 840 (PKT. PANG ET AL.) 18 August 1987 -----	

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 94/00718

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		CA-A-	2136330	25-11-93
		EP-A-	0641222	08-03-95
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		CA-A-	2112798	11-11-93
		EP-A-	0593754	27-04-94
EP-A-398143	22-11-90	AU-A-	5659090	29-11-90
		CA-A-	2016235	09-11-90
		JP-T-	4505761	08-10-92
		WO-A-	9013317	15-11-90
US-A-4687840	18-08-87	NONE		